CLONING OF A HYPOXIA INDUCIBLE FACTOR-1α AND CAR-VASCULAR HOMING PEPTIDE RECOMBINANT FUSION PROTEIN TEMPLATE TO PSECTAG2-VECTOR

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Syventävien opintojen kirjallinen työ
LK Panu Antinmaa

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Avainsanat: cell penetrating peptide, transcription factor, hypoxia, wound repair.

Tiivistelmä:

Diabeetikot ovat alttiita jalkojen haavaumille, johtuen suureksi osaksi perifeerisestä neuropatiasta, heikentyneestä angiogeneesistä ja vähentyneestä granulaatiokudoksen muodostuksesta sekä myös heikentyneen solusignaloinnin ja heikentyneiden kasvutekijävasteiden johdosta. Tämä tutkimusraportti kuvaa hypoksian indusoima tekijä-1α:n (Hypoxia Inducible Factor-1α, HIF-1α) sekä verisuoniin hakeutuvan CAR-peptidin cDNA:n kloonausta psectag2-vektoriin. Kloonaus suoritettiin, jotta voitaisiin tuottaa rekombinantti fuusioproteiini, jossa olisi liitettynä HIF-1α:n ja CAR-peptidi.

Kloonaustrategiamme perustui haavojen verisuoniin hakeutuvaan CAR-peptidiin, joka on tunnistettu selomalla peptidikirjastoja (sisältäen $1x10^9$ erilaista peptidiä). CAR-peptidi hakeutuu angiogeenisiin verisuoniin regeneroituvassa kudoksessa ja voi toimittaa siihen liitettyjä molekyylejä regeneroituvaan ihoon, luurankolihakseen ja jännehaavoihin jopa 200-kertaisina pitoisuuksina verrattaessa kontroilleihin. Lisäksi CAR-peptidi on hyvin voimakas solukalvon läpäisevä peptidi, joka leviää vaurioituneissa kudoksissa ja läpäisee solukalvoja. On osoitettu, että CAR-peptidiin liitetyt molekyylit tai rekombinantit proteiinit voidaan toimittaa soluihin.

HIF-1 on suoraan happipitoisuuden säätelemä dimeerinen transkriptiotekijä, joka säätelee angiogeneesille tärkeiden geenien ekspressiota soluissa ja kudoksissa. Jotta HIF-1α voisi lisätä angiogeneesille tärkeiden kasvutekijöiden pitoisuutta kohdekudoksissa, sen täytyy päästä kudoksen solujen sisäpuolelle läpäisemällä solukalvot.

Tutkimusryhmämme tulee käyttämään CAR-peptidin haavan verisuoniin hakeutumista ja kudosläpäisevyyttä hyväksemme, tuottaaksemme fuusioproteiinin, jossa on liitettynä stabiloitu ja/tai lyhennetty muoto HIF-1α:sta.

Kloonauksen jälkeen fuusioproteiinin templaatti-DNA:n sisältävä vektori lähetettiin proteiinituotantoa varten laboratorioon.

Contents

1. Aim of the study

The purpose of this study was to produce a recombinant fusion protein consisting of mutated (constitutively active) version of the transcription factor Hypoxia inducible factor-1α (HIF-1α) and the vascular homing peptide CAR. This fusion protein could theoretically improve circulation and oxygen delivery to ischemic wounds in target organ specific fashion by inducing angiogenesis in the regenerating tissues. Mutated HIF-1α has been shown to improve tissue perfusion in ischemic rabbit muscle using adenoviral delivery system (1).

2. Review of the Literature

2.1 Hypoxia inducible factor-1**α**

Hypoxia inducible factor-1α (HIF-1α) is one of the two proteins of the dimeric transcription factor HIF-1. HIF-1 is a heterodimer of HIF-1α and HIF-1β. The function of HIF-1 in cells is to trigger a cellular response and adaptation to hypoxia (low oxygen concentration). It achieves this by affecting the transcription of numerous genes that either help cells to survive in hypoxic conditions (adaptation) or increase the delivery of oxygen to the cellular environment by inducing formation of neovessels i.e. angiogenesis (homeostatic response). (2,3)

HIF-1 is a transcription factor that carries out its functions by up-regulating genes involved in cellular processes related to cellular survival in hypoxia, such as angiogenesis (vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β)), cell growth and survival (insulin-like growth factor-2 (IGF-2), transforming growth factor-α (TGF-α)), glucose metabolism (lactate dehydrogenase A, phosphoglycerate kinase 1, glucose transporters (GLUTs) 1,3), erythropoiesis (erythropoietin), iron metabolism (ceruloplasmin, transferrin), matrix metabolism (matrix metalloproteinases) and vascular tone (endothelin, heme oxygenase 1, nitric oxide synthase 2). (2,3)

HIF-1α and HIF-1β are both constitutively expressed and produced in all of our cells, but HIF-1α is immediately degraded after its production in normoxic conditions. —The mechanism behind the rapid degradation of HIF-1α is directly linked to oxygen; In normoxic conditions either one or both prolyl residues (amino acids 402 and 564) of HIF-1α are hydroxylated by prolyl-4-hyroxylases (PHD-4). Prolyl-4-hydroxylases use oxygen and α-ketoglutarate as substrates. This hydroxylation of prolyl residues allows the Von Hippel-Lindau (VHL) -tumor suppression protein to bind with HIF-1α. VHL then recruits E3-ubiquitin-protein ligase, which, in turn, ubiquitinates HIF-1α and directs it for proteosomal degradation inside the cells. Furthermore, the activity of HIF-1α is also regulated by hydroxylation of asparagine residue 803 by factor inhibiting HIF-1 (FIH-1). This process inhibits binding of transcriptional co-activators CBP and p300 to HIF-1α. (2,3)

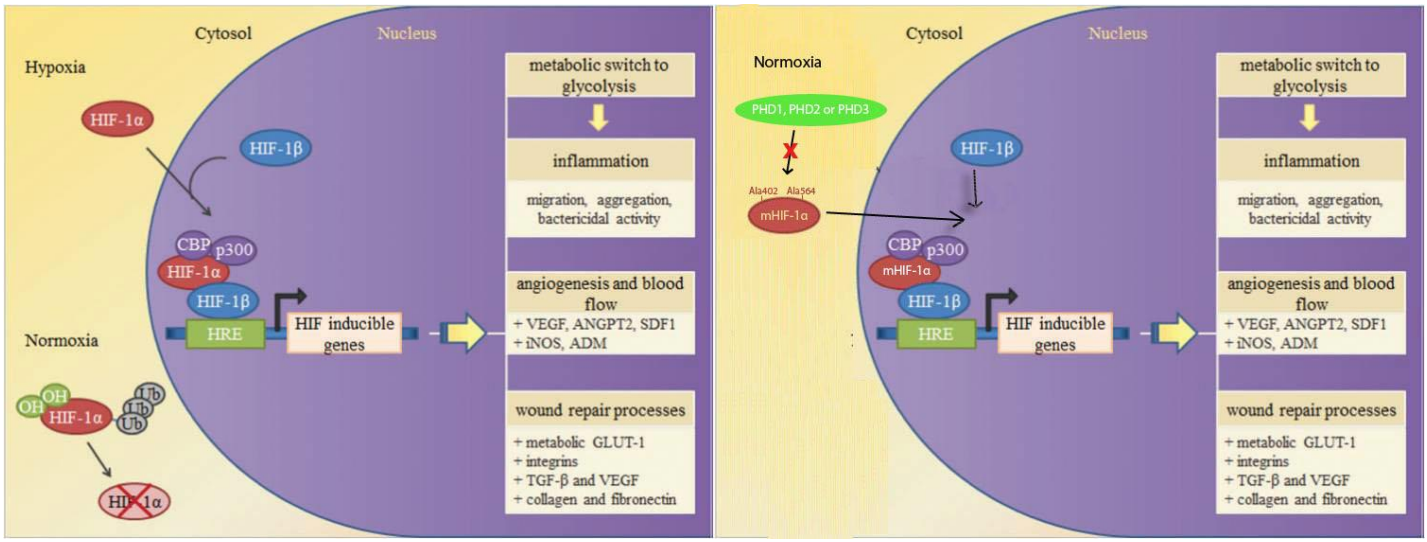

Fig. 1. Activation of HIF-1 and mutated HIF-1**α** (mHIF-1**α**). In hypoxic conditions HIF-1α is hydroxylated by prolyl-4-hydroxylases. This allows binding of Von Hippel-Lindau (VHL) tumor suppressor protein and results in ubiquination of HIF-1α. Ubiquination directs HIF-1α for proteosomal degradation.

In normoxic conditions HIF-1α is not hydroxylated, binds with HIF-1β and translocates into the nucleus. In the nucleus HIF-1 binds to genes that have hypoxia regulatory elements (HRE) in their promoter region and recruits coactivators such as CBP and p300. Formation of this complex induces expression of target genes.

mHIF-1α has proline residues 402 and 564 mutated to alanine residues. This prevents hydroxylation by PHD1, PHD2 or PHD3 and binding of VHL. mHIF-1α can bind with HIF-1β and translocate to the nucleus. In the nucleus HIF-1 recruits coactivators and induces expression of target genes. Image modified from (2).

HIF-1α is not degraded by above described mechanism in hypoxic conditions and can then bind and form heterodimers with HIF-1β. The heterodimeric HIF-1 translocates to the nucleus, where HIF-1 functions as a transcription factor for large number of its target genes. HIF-1 binds DNA and induces the transcription of genes involved in oxygen and vascular homeostasis (please see below). (2,3)

HIF-1 regulates genes that contain the hypoxia response element (HRE) in their promoter region. These genes mediate a multitude of intracellular, paracrine and endocrine functions all aiming at providing survival in low oxygen conditions, i.e. in hypoxia. One group of HIF-1 target genes regulate cellular glucose metabolism. (2-5)

As a result of what is described above, mutation of prolyl residues 402 and 564 and the asparagine residue 803 to alanines could possibly stabilize HIF-1α and thus, potentially provide HIF-1 driven constitutive transcriptional activity even in normoxic conditions. (1)

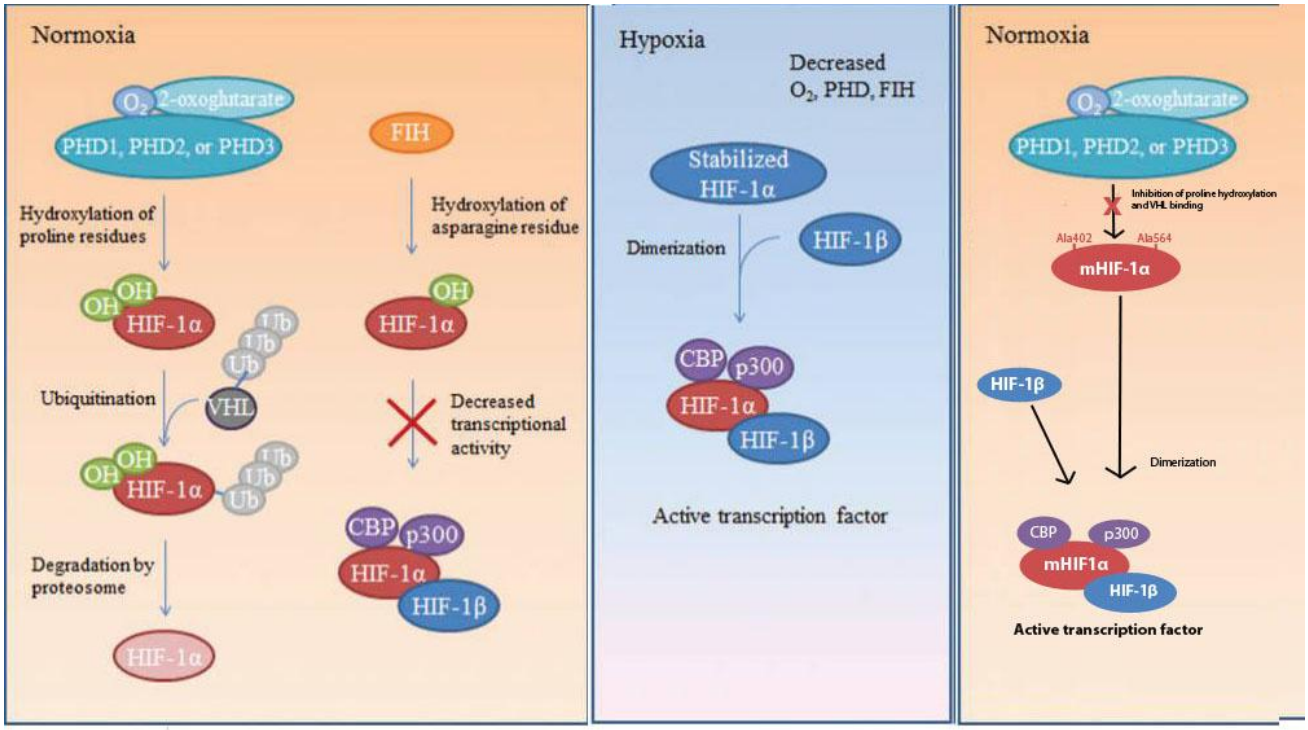

Fig. 2. Illustration of HIF-1**α** and mutated HIF-1**α** (mHIF-1**α**) being regulated in hypoxic and normoxic conditions. Normoxia results in prolyl residues 402 and 564 of HIF-1α being hydroxylated by PHD1, PHD2 or PHD3. Hydroxylation of these residues allows binding of Von Hippel-Lindau (VHL) tumor suppressor protein. VHL recruits a ubiquitin-protein ligase complex that ubiquinates HIF-1α and directs it for proteosomal degradation. Factor inhibiting HIF-1 hydroxylates asparagine residues within HIF-1α which inhibits binding of coactivators and results in decreased activity.

During hypoxia activity of PHD1, PHD2, PHD3 and FIH are decreased which allows HIF1α to bind with HIF1β. HIF-1 then translocates into the nucleus and recruits transcriptional coactivators such as p300 and CBP. This allows expression of target genes.

In normoxic conditions mHIF-1α is not hydroxylated by PHD1,PHD2 or PHD3 and can therefore bind with HIF-1β. HIF-1 then translocates to the nucleus and recruits coactivators such as CBP and p300 to form an active transcription factor. This transcription factor can induce expression of target genes. Image modified from (2).

2.2 CAR-homing peptide

The CAR wound-homing peptide (full sequence CARSKNKDC) was identified by *in vivo phage display* in the peptide library screen for peptides that home to angiogenic blood vessels in regenerating tissues. (6,7)

The CAR peptide has been shown to specifically bind to newly formed angiogenic blood vessels. This peptide has been shown to be capable of delivering systemically administered, therapeutic recombinant proteins, such as anti-fibrotic protein decorin (and human serum albumin), that are beneficial to wound healing, to wounds by targeting the cargo attached to CAR to angiogenic blood vessels being formed at the site of injury. (6,8). —Furthermore, the CAR peptide is a cell penetrating peptide that extravasates into the early granulation tissue, internalizes, and delivers cargo inside the cells (9).

So far, it has been shown that the cell-penetrating CAR peptide is capable of delivering fluorescein, bacteriophage, extracellular matrix protein decorin and serum albumin into the nucleus of the cells (6,7,9). Thus, these properties make CAR peptide a plausible candidate to deliver transcription factors into the cells in target organ specific fashion (6,7,9).

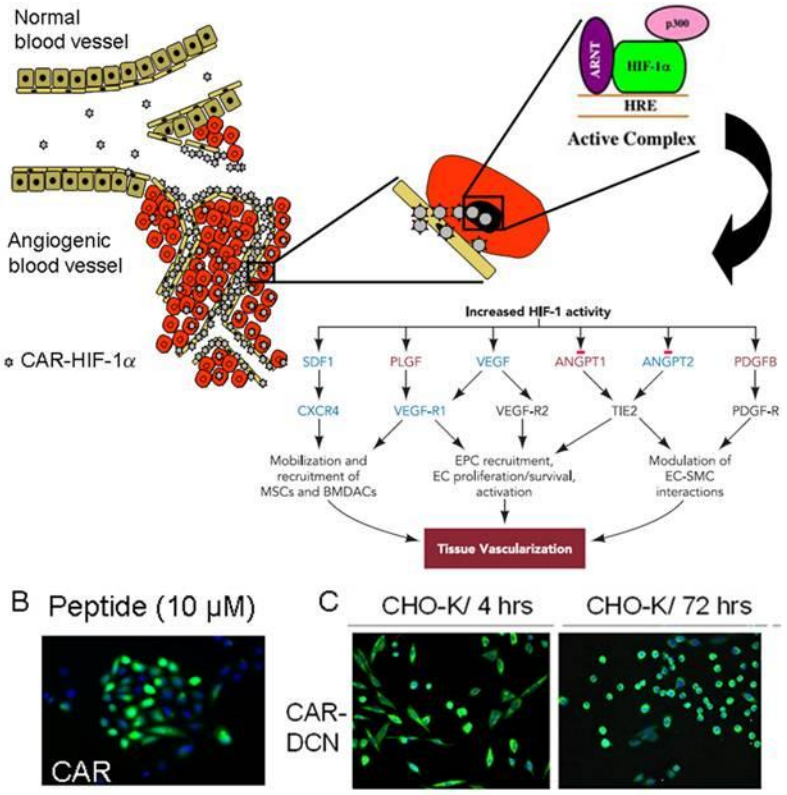

Fig. 3. Strategy for CAR-HIF-1α in diabetic wounds. HIF-1α will be expressed as a fusion protein with vascular targeting peptide CAR. CAR will deliver HIF-1α to the angiogenic vasculature forming at injured tissue, extravasate and penetrate into the cells. HIF-1α will turn on large number of angiogenic growth factors, such as VEGF and erythropoietin (EPO). B) Demonstration of CAR peptide being a potent cell penetrating peptide and C) extracellular protein decorin can be internalized into the CHO-K cells by CAR.

2.3 Wound repair

Wound repair can be divided to five phases: acute phase, inflammation, proliferation, maturation and remodeling. In the acute phase, a blood clot is formed and growth factors and chemokines are released from extracellular matrix and intracellular vesicles in platelets. The inflammation phase begins after several hours and is characterized by extravasation and migration of neutrophiles, macrophages, endothelial cells and fibroblasts to the wound site. Especially the macrophages are involved in secreting additional growth factors that induce re-epithelialization, angiogenesis and fibroblast proliferation. In the proliferation phase, more cells migrate to the wound site and begin to proliferate. Fibroblasts in the wound site deposit new extracellular matrix. Collagen deposition is directly related to wound oxygen tension and perfusion. Maturation phase consists of resolution of cell proliferation and

extracellular matrix deposition as well as inflammation. In the remodeling phase, extracellular matrix and cells are reorganized to a stronger and more durable state and the scar formation takes place as an outcome of this process. (10)

2.4 Diabetic ulcers

There are over half a million diabetics in Finland. Diabetic patients are prone to foot problems caused by complications of diabetes: peripheral arterial disease (PAD) and peripheral neuropathy. About 9-23% of diabetic patients are estimated to have peripheral arterial disease. Of diabetic foot ulcers 10% have only PAD as an etiologic factor and 50% ischemia together with peripheral neuropathy (11). The lifetime prevalence of foot ulcer in diabetics is 15% (12).

Wound healing in diabetes is impaired because of enzyme and protein dysfunction resulting from hyperglycemia, impaired oxygen delivery and disturbances in the immune system (10).

Wound repair			
Acute phase (minutes to hours)	Inflammation (hours to days)	Proliferation (days to weeks)	Remodeling (weeks to years)
Disruption of vascular endothelium Extravasation of blood constituents Activation of platelets Release of growth factors involved with chemotaxis, angiogenesis and re-epithelialization	Arrival of inflammatory cells to wound site Activation of monocytes to tissue macrophages Release of additional cytokines and growth factors (e.g. transforming growth factor-β, platelet derived growth factor, fibroblast growth factor, epidermal growth factor) by macrophages Phagocytosis of debris, bacteria and breakdown tissue by polymorphonuclear neutrophils and macrophages Release of vasodilative signaling molecules/inflammatory factors by macrophages	Proliferation of keratinocytes in wound edges and dermal appendages (e.g. hair follicles, sweat glands and sebaceous glands); re-epithelialization Deposit of new extracellular matrix by fibroblasts i.e. Granulation Formation of new blood vessels (i.e. angiogenesis) Migration and proliferation of fibroblasts	Equalization of collagen formation and degradation Reorganization of deposited extracellular matrix

Fig. 4. Phases of wound repair.

3. Materials and methods

A full-length human mutated HIF-1α (mHIF-1α) cDNA was kindly provided by Dr. L. Poellinger (Karolinska Institute, Stockholm, Sweden). PCR of full length mHIF-1α cDNA was performed using following primers; HifBamH1forw. (acgtggatccatggagg gcgccggcgg c) and HifEcoRIrev (gacgaattccgagccgttaacttgatccaaagc). Primer Tm was calculated using Finnzymes Tm calculator (http://www.thermoscientificbio.com/webtools/tmc/). The PCR product was purified with DNA purification kit (Qiagen, Venlo, Netherlands). The purified PCR product (after confirming the right size by gel electrophoresis) was digested using restriction enzymes BamH1 and EcoR1 (NEB, New England Biolabs, Cambridge, MA, USA). The digested product was purified using DNA purification kit (Qiagen, Venlo, Netherlands) according to protocol.

The pcDNA3.1/myc-his-C-expression vector (Invitrogen, Carlsbad, CA, USA) with following existing construct: BamH1 restriction site+decorin+EcoR1-restriction site+CAR-peptide+trombin cleavage site+HisTag (described previously in (8)) was digested with BamH1 and EcoR1 restriction enzymes to cut decorin cDNA out of the vector. The digested products were run on 1%-agarose gel with DNA-ladder and the band matching the right size (linear DNA) of EcoR1+CAR-peptide+thrombin cleavage site+HisTag in pcDNA3.1 was cut from gel and purified by DNA agarose gel extraction kit (Qiagen, Venlo, Netherlands) according to protocol.

mHIF-1α PCR product was ligated to EcoR1+CAR-peptide+thrombin cleavage site+HisTag in pcDNA3.1 using T4 DNA ligase (NEB, New England Biolabs, Cambridge, MA, USA) according to overnight ligation protocol at 16 °C. Ligation product was used to transform One Shot TOP10F' Competent Cells (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Cells were spread on LB agar ampicillin plates and plates were incubated overnight at 37 °C. Colonies from LB plates were selected for incubation at +37°C in LB (supplemented with ampicillin) medium. Glycerol stocks were made of incubated medium and vectors purified using Miniprep kit (Qiagen, Venlo, Netherlands).

Constructs were sequenced using following sequencing primers: T7 promoter (taatacgactcactataggg), BGH Reverse (tagaaggcacagtcgagg), Hif Seq sec 1(cagaaatggcccagtgaga), Hif Seq sec 2 (gattttctcccttcaacaaacag), Hif Seq sec 3 (aaaactgtttgctgaagacacaga). Primers were chosen 400 base pairs apart from each other to cover the whole construct. The OligoPerfect™ Designer (https://tools.lifetechnologies.com/content.cfm?pageid=9716&icid=fr-oligo-6?CID=fl-oligoperfect) was used for their optimal design.

PCR of mHIF-1α+CAR-peptide+thrombin cleavage site+HisTag-construct was performed using primers HifSfilforw. (ggcccagccggcc gagggcgccggcggcgag) and HisTagXhoIrev (tctagactcgagttagtgatggtg). The PCR-product was purified using DNA purification kit according to protocol (Qiagen, Venlo, Netherlands). The purified PCR product was digested (after confirming right size with gel electrophoresis) with

restriction enzymes SfiI and XhoI (NEB, New England Biolabs, Cambridge, MA, USA). The digested product was purified using DNA purification kit according to protocol (Qiagen, Venlo, Netherlands).

pSecTag2-vector (Invitrogen, Carlsbad, CA, USA) was digested with restriction enzymes SfiI and XhoI (NEB, New England Biolabs, Cambridge, MA, USA). Digestion with SfiI and XhoI cut out most of restriction sites in the multiple cloning site of pSecTag2. The reaction solution ran on 1%-agarose gel with DNA ladder. The band consistent with the size of the linearized, digested vector was cut from gel and purified using agarose gel extraction kit according to the manufacturer´s protocol (Qiagen, Venlo, Netherlands).

HIF-1α+CAR-peptide+thrombin cleavage site+HisTag-construct was ligated to psectag2-vector using T4 DNA-ligase (NEB, New England Biolabs, Cambridge, MA, USA) according to overnight ligation protocol. Ligation product was used to transform One Shot TOP10F' Competent Cells (Invitrogen, Carlsbad, CA, USA). Cells were spread on LB ampicillin-agarose plates and plates were incubated overnight at +37°C. Colonies were selected for incubation at +37°C in LB-ampicillin medium. After incubation glycerol stocks were made of incubated medium and minipreps were made using Miniprep kit (Qiagen, Venlo, Netherlands). Products were sequenced using sequencing primers described above.

HIF-1α+CAR-peptide+thrombin cleavage site+HisTag-construct in psectag2-vector was sent for protein production in mammalian cells.

Figure 5. Schematic representation of domain structure of CAR-HIF-1α-cDNA fusion construct. NTAD (N-terminal transactivation domain); CTAD (C-terminal transactivation domain); NLS (nuclear localisation signal). See text for description.

4. Results

Sequencing confirmed the cloning product to be consistent with the insert HIF-1α+CAR+Trombin cleavage site+HisTag bein incorporated into psectag2-vector. The construct was sent for protein production in baculovirus expression system.

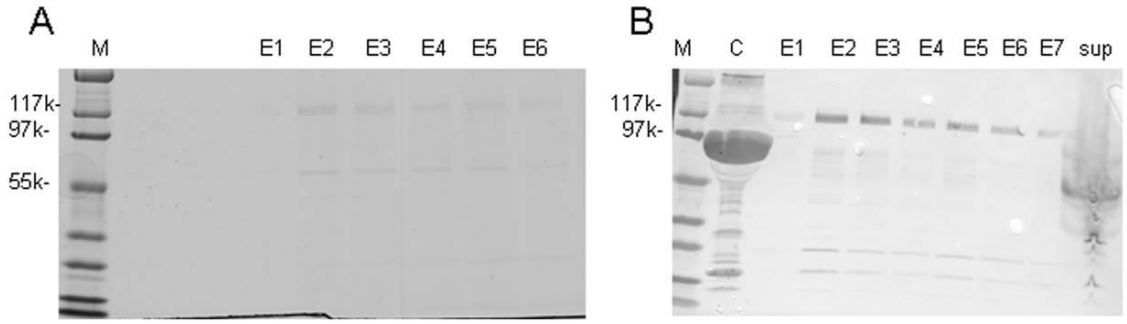

Figure 6. Production of recombinant CAR-HIF-1α fusion proteins in the baculovirus system. The recombinant protein was expressed in baculovirus expression system, purified on a Ni-NTA-column, separated on gradient SDS-PAGE gels. (A) SDS-PAGE gel was stained by Coomassie. (B) Western blot analysis: proteins were transferred to nitrocellulose membrane and detected with a monoclonal anti-6-histidine tag antibody. E1 – E6 eluates after the purification; C –positive control; sup - supernatant from uninfected culture.

5. Discussion

Diabetic wounds are difficult and expensive to treat. They result from protein and enzyme dysfunction that is caused by hyperglycemia, impaired oxygen delivery (caused by micro- and macrovascular changes) and disturbances in the immune system. (10) Diabetic foot is also more susceptible to trauma because of peripheral neuropathy caused by diabetes (11). Improving oxygen delivery to diabetic wounds could improve wound healing and reduce the number of patients with chronic wounds, wound infections and amputations in diabetics.

Gene therapy with mHIF-1α delivered by adenoviral transfection vector has been shown to improve wound healing in a rabbit ischemic muscle model (1). As a master gene switch for angiogenic program HIF-1 can upregulate multiple genes involved with angiogenesis (2). A mutated HIF-1α and CAR-peptide fusion protein should accumulate in the newly formed blood vessels in the wound and potentially improve wound healing by stimulating angiogenesis and improving oxygen delivery.

Recombinant HIF-1 has been successfully produced in bacterial cells (13). Our aim is to produce HIF-1 coupled to wound homing and cell penetrating peptide CAR in baculovirus essentially in the same fashion as described in E.coli by Van de Sluis (13). In addition to full-length HIF-1α (1-826), we will generate following variants; short form of HIF-1α (1-390) (14,15), as the truncated version of HIF-1α has been more active than the native, full-length HIF-1α and has been used in clinical gene therapy-trials in humans (14,15). The latter will consist of the native DNA-binding and dimerisation subunits of the HIF-1α, but is devoid of the natural transactivation domains, which are replaced by either one of the two independent, strong, oxygen-independent, constitutively active transactivation domains; 1) herpes simplex virus VP16 transactivation domain (16) or 2) 11 tandem copies of FDTDL, a minimal, critical region from a very potent transactivation domain of β-catenin (17). Both VP16 and [FDTDL]x11 have been used successfully for similar outcome and have been shown to provide stronger transactivating domains than the native HIF-1α transactivation

domains does (16,17). CAR will be juxtaposed to the transactivation domain after a short flexible linker (Fig. 6). In addition of being more active than the native HIF-1α, the truncated CAR-HIF-1α fusion-protein should be substantially smaller than the full-length protein. We anticipate that, the probability of robust protein production in native conditions is increased substantially by this reduction in size of the recombinant protein.

With recombinant CAR- vascular homing peptide fused to HIF-1α, we hope to deliver HIF-1α inside of cells in wounds in target organ specific fashion. After internalization of the recombinant protein to the cells by the cell penetrating capabilities of CAR, the recombinant mHIF-1α can dimerize with HIF-1β and translocate into the nucleus. Inside the nucleus HIF-1 will induce transcription of target genes e.g. VEGF. With this, we hope to stimulate angiogenesis and improve oxygen delivery and ultimately the outcome of wound healing in diabetic patients in disease-specific fashion.

6. References

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7. Supplemental material

HIF1α+CAR+Thrombin cleavage site+HisTag sequence

AGAGGGCGCCGGCGGCGAGAACGAGAAGAAAAATAGG

ATGAGTTCTGAACGTCGAAAAGAAAAGTCTAGAGATGCAGCAAGATCTCGGCGAAGCAAAGAGTCTGAAG

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TCCAAG

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CACCACA

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GATTGCAT

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CAAGGAGC

CTTAACCTGTCTGCCACTTTGAATCAAAGAAATACTGTTCCTGAGGAAGAATTAAACCCAAAG
ACAATAG

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TGCTGGGGCAGTCAATGGATGTGAGTGGATTACCACAGCTGACCAGTTACGATTGTGAAGTT
AATGCTCC

CATACAAGGCAGCAGAAACCTACTGCAGGGTGAAGAATTACTCAGAGCTTTGGATCAAGTTA
ACGGCTCG

GAATTCTGTGCACGTTCGAAGAACAAAGATTGCGTCGACCTGGTGCCGCGCGGCAGCTCGCA
TCACCATC

ACCATCACTAACTCGAG

Protein sequence

E G A G G E N E K K N R Met S S E R R K E K S R D A A R S R R S K E S E V F Y E L A H Q L P
L P H N V S S H L D K A S V Met R L T I S Y L R V R K L L D A G G L D S E D E Met K A Q Met
D C F Y L K A L D G F V Met V L T D D G D Met V Y I S D N V N K Y Met G L T Q F E L A G H
S V F D F T H P C D H E E Met R E Met L T H R N G P V R K G K E L N T Q R S F F L R Met K
C T L T S R G R T Met N I K S A T W K V L H C T G H I H V Y D T N S N Q P Q C G Y K K P P
Met T C L V L I C E P I P H P S N I E I P L D S K T F L S R H S L D Met K F S Y C D E R I T E L
Met G Y E P E E L L G R S I Y E Y Y H A L D S D H L T K T H H D Met F T K G Q V T T G Q Y R
Met L A K R G G Y V W V E T Q A T V I Y N T K N S Q P Q C I V C V N Y V V S G I I Q H D L I
F S L Q Q T E S V L K P V E S S D Met K Met T Q L F T K V E S E D T S C L F D K L K K E P D A
L T L L A A A A G D T I I S L D F G S D D T E T E D Q Q L E D V P L Y N D V Met F P S S N E K
L N I N L A Met S P L P S S E T P K P L R S S A D P A L N Q E V A L K L E S S P E S L G L S F T
Met P Q I Q D Q P A S P S D G S T R Q S S P E P N S P S E Y C F D V D S D Met V N V F K L
E L V E K L F A E D T E A K N P F S T Q D T D L D L E Met L A A Y I P Met D D D F Q L R S F
D Q L S P L E S N S P S P P S Met S T V T G F Q Q T Q L Q K P T I T A T A T T T A T T D E S K
T E T K D N K E D I K I L I A S P S S T Q V P Q E T T T A K A S A Y S G T H S R T A S P D R A G
K R V I E Q T D K A H P R S L N L S A T L N Q R N T V P E E E L N P K T I A S Q N A Q R K R K
Met E H D G S L F Q A A G I G T L L Q Q P G D C A P T Met S L S W K R V K G F I S S E Q N
G T E Q K T I I L I P S D L A C R L L G Q S Met D V S G L P Q L T S Y D C E V N A P I Q G S R
N L L Q G E E L L R A L D Q V N G S E F C A R S K N K D C V D L V P R G S S H H H H H H
Stop